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Development and Characterization of 15 Polymorphic Microsatellite Markers for a Highly Adaptive and Wide-range Frog (*Microhyla fissipes*)

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Abstract In order to analyze population genetic structure at multiple spatial scales, microsatellite loci were developed for the ornamented pygmy frog (*Microhyla fissipes*), and 15 polymorphic microsatellite loci were successfully screened from 105 individuals, of which 82 from four populations distributed in the Sichuan Basin and 23 from the Sangzhi population in western Hunan. Five loci were found to deviate significantly from Hardy-Weinberg equilibrium in one to three populations, probably due to small sample size or null alleles. The average number of alleles in all loci was 8.5, ranging from 4 to 13, and the observed and expected heterozygosity ranged from 0.26 to 0.90 and 0.63 to 0.90, respectively. The Sangzhi population and the remaining four populations can be clearly separated using Bayesian clustering methods, showing that the genetic structure of *M. fissipes* was probably affected by the topography, especially mountain barriers. These polymorphic microsatellite loci could be used for further study on the landscape genetics of this highly adaptive and widely distributed species.

Keywords *Microhyla fissipes*, microsatellite, genetic diversity, population genetics

1. Introduction

Microhyla fissipes is a small-sized frog with a narrow mouth, and inhabits soil pits or grass thickets on plains or hills at altitudes below 1400 m, where it eats termites and ants among the litter. The species is highly adapted to various stressful environments, as it could breed in small ponds or even temporary puddles shortly after rains and is capable of laying multiple egg clutches during the long breeding season from March to August. The eggs may hatch relatively soon (~ 24 hours) after fertilization and the tadpoles can complete metamorphosis within 20–30 days (Fei *et al*., 2009), thus it may be considered a model for studying the plasticity of breeding behaviors in stressful environments.

Based on recent mtDNA analyses, the name *M. fissipes* was revived by Matsui *et al*. (2005) for the populations of the species *M. ornata* sensu lato by Fei *et al*. (2009) in a wide range from eastern mainland Asia to Taiwan, China, whereas the name *M. ornata* is restricted to the South Asian populations and a new name *M. okinavensis* was nominated for the Ryukyu populations (Matsui *et al*., 2005). Some other wide-ranging frog species have been proven to include several cryptic species or different lineages, such as *Rana chensinensis* (Zhou *et al*., 2012) and *Feirana quadranus* (Wang *et al*., 2012). *M. fissipes* was found to have high chromosome diversity and karyotype diversity across geographical ranges (Qian and Zhu, 2000). As a common and widely-distributed species, *M. fissipes* may prove to be a useful model to study adaption or speciation of amphibians across geographic regions.

For a better understanding of its gene flow across urban or mountain landscapes, phylogenetic structure, and mating system during stressful or good seasons, some useful bi-parental nuclear markers-microsatellite were developed in this study. This is the first attempt to isolate microsatellite loci and characterize genetic structure for this species, which will promote further research on this species.

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2. Materials and Methods

2.1 Specimens We used 105 samples of *M. fissipes* from four populations around the Sichuan Basin and one population from Sangzhi in Hunan (Table 1). Voucher specimens were deposited in the Chengdu Institute of Biology, Chinese Academy of Sciences, Chengdu, China.

Table 1 Voucher specimens of the five populations used in this study.

Population	Voucher No.	GPS position
Anxian (AX, Sichuan)	20110802001–020	31°21′15.876″N, 104°11′32.100″E
Jianyang (JY, Sichuan)	20120707001–005	30°11′1.824″N, 104°10′13.259″E
	20120707007	
	201207009–018	
Nanjiang (NJ, Sichuan)	20110962–983	32°12′37.476″N, 106°29′35.628″E
Pujiang (PJ, Sichuan)	20110908001–024	30°6′54.720″N, 103°18′10.152″E
Sangzhi (SZ, Hunan)	20120621001–002	29°14′30.228″N, 110°6′2.016″E
	20120621004–010	
	20120621012–014	
	20120621016–026	

2.2 Laboratory experiment

Genomic DNA extraction: Total genomic DNA was extracted from muscle tissues (preserved in 95% ethanol) using the EasyPure Genomic DNA Extraction Kit (Beijing TransGen Biotech Co., Ltd.).

Sequencing of fragments containing tetranucleotide repeats: A microsatellite-enriched *M. fissipes* genomic library was constructed according to Fast Isolation by AFLP of Sequences Containing Repeats (FIASCO) protocol by Zane *et al*. (2002).

Briefly, DNA from one *M. fissipes* specimen (specimen code: CIB20110802005) was simultaneously digested with *Mse*I restriction enzyme (New England BioLabs) and ligated to *Mse*I AFLP adapters (MseIA: 5'-TACTCAGGACTCAT-3', MseIB: 5'-GACGATGAGTCCTGAG-3') (Vos *et al*., 1995). The digestion-ligation mixture was then diluted (1:10) and directly amplified with AFLP adapter-specific primers (*Mse*I-N). The product was purified, denatured, and hybridized to a 5'-biotinylated $(GATA)_6$ probe at 68 °C for 3 h, and then the fragments containing microsatellite sequences were captured by streptavidin-coated magnetic beads (Promega). Non-specific DNA was removed by four nonstringency washes and four stringency washes. DNA fragments were separated from the bead-probe complex by two denaturation steps (at 95 °C for 5 min and with 0.15 M NaOH for 20 min).

PCR was conducted using *Mse*I-N primers to make fragments double-stranded. Fragments ranging from 400 to 1000 bp were excised from 1% agarose gel, and then ligated with pMD19-T vector (TaKaRa) at 16 °C for 5 h and transformed to Trans5α chemically competent cells (TransGen Biotech). The cells were placed onto LB agar plates containing ampicillin, IPTG and X-gal, and then incubated at 37 °C for 10 h. After that, we chose those single white colonies and transferred them into LB containing ampicillin.

The cloned fragments were amplified using three primers: the universal primers of M13 and the $(GATA)_6$ tandem repeat primer. Only those sequences that have enough long flanking regions and tetranucleotide repeats were used for designing primers.

Design of primers for microsatellite loci and test of their utility: Primers were designed using Primer Premier 6.0 (Premier, Canada) and Oligo 7.0 (Rychlik, 2007). The developed primers were amplified on 16 samples randomly selected from the five populations. The amplification reaction used 10 μl PCR mixture containing approximately 0.7 ng of diluted genomic DNA, 0.4 μM of each primer and 4 μl EasyTaq PCR SuperMix (TransGen Biotech). Amplifications were performed using the following cycling profiles: initial denaturation at 94 °C for 3 min; followed by 35 cycles at 94 °C for 30 s, at annealing temperature (Ta) for 40 s (Table 2), at 72 °C for 1 min; and then a final elongation step at 72 °C for 12 min. For the primers that could successfully yield clear target products, the forward primer of each pair was labeled with one of the fluorescent dye FAM, HEX or Rox, and ran the PCR-products on an ABI-3730xl sequencer.

Analyses of microsatellite data: The electropherograms of microsatellites were scored with GeneMarker v1.85 (SoftGenetics LLC). The number of alleles (*A*), polymorphism information content (PIC), observed heterozygosity (*H*o) and expected heterozygosity (H_E) were calculated with CERVUS v3.0 (Kalinowski *et al*., 2007). The tests for deviation from Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium (LD) were performed in GENEPOP v4.0 (Rousset, 2008). The software Micro-checker 2.2.0 (van Oosterhout *et al*., 2004) was used to test for technical artefacts such as null alleles, stuttering and large allele dropout.

Population genetic structure was analyzed using the program STRUCTURE v2.3, which implements a model-based clustering method for inferring population structure using genotype data consisting of unlinked

Table 2 Characterization of the 15 polymorphic microsatellite loci developed for *Microhyla fissipes*.

Locus	GenBank accession No.	Primer sequences	Repeat motif	Size of alleles	*T*a	PIC	A
CIBmf01	KF053106	F:5'-CTCCCAGAATGTTGTTATAGTG-3' R:5'-GTACTGAAGAATTGTCCCTCT-3'	$(TAGA)_{20}$	273–309	59	0.79	10
CIBmf02	KF053107	F: 5'-TGGTAATCTTCAGGTCATCT-3' R: 5'-CTGTTCCATCTGTCTTTCG -3'	$(TCTA)_{16...}$ $(TATC)_{12}$	327–375	56	0.80	13
CIBmf03	KF053108	F:5'-GTCCTCAGCGTTCTAGTG-3' R:5'-GTAAACATCTAGGAAGTGACAC-3'	$(AGAT)_{13...}(T)_{10}$	262–285	56	0.63	9
CIBmf04	KF053109	F:5'-GGCTGACACATGACGATAG-3' R:5'-GTCCCTCTCATATTATCTCCAT-3'	$(TCTA)_{14}$	232–276	59	0.78	12
CIBmf05	KF053110	F:5'-GGGTACACATAGCATTGAAA-3' R:5'-ACACCATAAATCCCAGTTGA-3'	$(ATAG)_{12}$	336–352	58	0.59	5
CIBmf06	KF053111	F: 5'-GGAAATATCGGTTCGGTTT-3' R: 5'-TTCGGTCTGTCTAAACTTGT-3'	$(ATAG)_{21}$	240–304	58	0.81	24
CIBmf07	KF053112	F:5'-CTGCTGGTGAACCTTACA-3' R:5'-TGTTAGTGCTGAGGTTGATT-3'	$(TCTA)_{18}$	311–407	56	0.73	20
CIBmf08	KF053113	F:5'- ATAGTGACTACCAGCAGGA-3' R:5'-TGCCTACTCCATCTATCCA-3'	$(TAGA)_{13}$	100–148	56	0.61	12
CIBmf09	KF053114	F:5'-TGTTAGGCGTCCAAATAGA-3' R:5'-CCGTAGTTCTGTCAGTCC-3'	$(ATAG)_{14}$	175–227	56	0.65	12
CIBmf10	KF053115	F:5'-ATGTGGAGCTTCTCATTCAT-3' R:5'-GGCCATGATTGCATTCCA-3'	$(TATC)_{29}$	221–373	58	0.85	29
CIBmf11	KF053116	F:5'-TACACATACGCCTCTAAGC-3' R:5'-GCAAGCAATGTATCCAGTC-3'	$(TATC)_{15}$	200–224	58	0.61	7
CIBmf12	KF053117	F:5'-CCAACTATATCCTCCTGTCTG-3' R:5'-GCCACAACACTCACAACT-3'	$(ATCT)_{15}$	120–180	56	0.47	6
CIBmf13	KF053118	F:5'-CGACCAAATCAAGGAAGTAG-3' R:5'-CAACCGAGTGCTGGAAAT-3'	$(CTAT)_{14}$	182–224	58	0.46	7
CIBmf14	KF053119	F:5'-TTAGCCGATCTCCAGACTT-3' R:5'-CAACATTCCATTGCCATCA-3'	$(TATC)_{12}$	246–292	58	0.75	12
CIBmf15	KF053120	F:5'-ATCTCCATAGAGCAACACAT-3' R:5'-GCGTATTGACCAAATTATGC-3'	$(TAGA)_{18}$	231–263	56	0.66	9

(Continued Table 2)

Locus	AX (n = 20)			JY (n = 16)			NJ (n = 22)			PJ (n = 24)			SZ (n = 23)		
	H_E	H_O	F_{IS}	H_E	H_O	F_{IS}	H_E	H_O	F_{IS}	H_E	H_O	F_{IS}	H_E	H_O	F_{IS}
CIBmf01	0.82	0.70	0.16	0.86	0.94	0.72	0.86	0.91	0.10	0.84	0.75	**0.02**	0.79	0.87	0.72
CIBmf02	0.81	0.80	0.07	0.85	0.81	0.65	0.86	0.86	0.71	0.86	0.92	0.83	0.82	0.78	0.34
CIBmf03	0.67	0.80	0.32	0.80	0.81	1.00	0.78	0.73	0.67	0.66	0.63	0.49	0.50	0.48	0.69
CIBmf04	0.74	0.65	0.22	0.86	0.81	0.54	0.75	0.77	0.99	0.88	0.92	0.98	0.90	0.91	0.17
CIBmf05	0.71	0.50	0.05	0.54	0.25	**0.00**	0.56	0.50	0.28	0.72	0.46	**0.01**	0.75*	0.09*	**0.00***
CIBmf06	0.88	1.00	0.80	0.88	0.88	0.95	0.91	0.86	0.26	0.84	0.71	0.07	0.83	0.87	0.57
CIBmf07	0.65	0.65	0.87	0.79	0.69	0.16	0.84	0.64	**0.02**	0.81	0.75	0.45	0.80	0.83	0.50
CIBmf08	0.63	0.70	0.90	0.68	0.63	0.53	0.57	0.64	0.92	0.73	0.75	0.17	0.74	0.87	0.13
CIBmf09	0.77	0.35	**0.00**	0.53	0.50	0.27	0.82	0.27	**0.00**	0.81	0.63	0.07	0.67	0.83	0.25
CIBmf10	0.83	0.90	0.83	0.86	1.00	0.97	0.89	0.95	0.78	0.88	0.88	0.35	0.94	0.91	0.53
CIBmf11	0.66	0.75	0.48	0.72	0.94	0.51	0.81	0.73	0.30	0.54	0.58	0.74	0.63	0.57	0.22
CIBmf12	0.57	0.70	0.41	0.77	0.75	0.56	0.33	0.36	1.00	0.61	0.63	0.56	0.40	0.35	0.13
CIBmf13	0.49	0.60	1.00	0.51	0.50	1.00	0.59	0.59	0.08	0.51	0.33	0.11	0.63	0.70	0.54
CIBmf14	0.79	0.75	0.57	0.79	0.81	0.37	0.86	0.77	**0.04**	0.82	0.83	0.62	0.78	0.83	0.82
CIBmf15	0.69	0.75	0.99	0.71	0.81	0.99	0.81	0.91	0.37	0.80	0.79	0.53	0.63	0.52	0.24

The primer pair, annealing temperature (T_a, °C), repeat motif, number of alleles (A), mean polymorphism information content (PIC), and size range of alleles (bp) are based on all individuals (n = 105). The expected (H_E) and observed (H_O) heterozygosities, and exact P-values by the Markov Chain method of Hardy-Weinberg test (F_{IS}) are reported. * means that locus CIBmf05 could be amplified in only 11 individuals from the Sangzhi population.

markers (Pritchard *et al*., 2000; Falush *et al*., 2003, 2007). Simulations were run using a burn-in period of 10 000 sweeps followed by 100 000 MCMC iterations. This was a sufficient number of iterations to guarantee convergence, as evidenced by the values of likelihood that was printed by the program. Admixture ancestry and correlated allele frequency models were used without prior knowledge of genetic information. The number of possible clusters (*K*) tested was set from 1 to 10 (*i.e*., gene pools assumed), and 50 independent runs were repeated to check for consistency of results (Noble *et al*., 2010). Twenty independent runs with the highest LnP (D) were averaged to estimate *K*. The true *K* was selected using the an hoc statistic Delta (*K*) based on the rate of change in the log probability of data between successive K values (Evanno *et al.*, 2005).

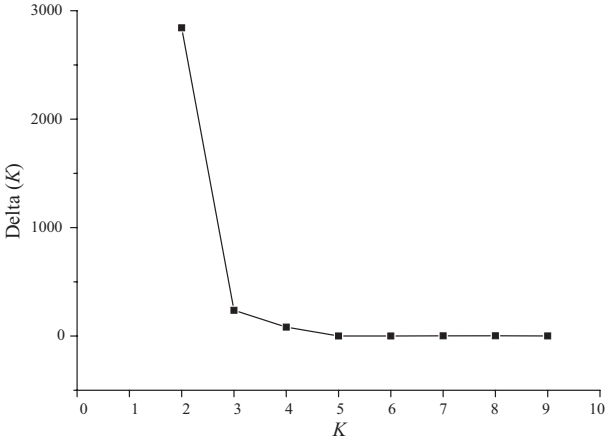

Figure 1 Bayesian STRUCTURE clustering results based on microsatellite genotypes among the five *Microhyla fissipes* populations. Delta (*K*) values as a function of *K* based on 50 runs, indicating the most likely number of two genetic clusters.

3. Results

3.1 Microsatellite loci In total, 220 colonies were sequenced and 194 sequences were found containing tetranucleotide repeats, of which 42 sequences had simple repeating motifs and enough flanking regions, and could be used to design primers. A total of 15 loci were successfully amplified in 105 individuals from the five populations (Table 2).

The average number of alleles in all loci for all five populations is 8.5, ranging from 4 to 13, and the observed and expected heterozygosity range from 0.26 to 0.90 and from 0.63 to 0.90, respectively (Table 2).

Significant linkage disequilibrium ($P < 0.05$) was detected in only 39/524 comparison pairs in the five populations. Results from Micro-Checker suggested null alleles may be present at the locus CIBfm05 due to stuttering. After Bonferroni correction for multiple comparisons (Holm, 1979; Rice, 1989), five loci were found to be deviated from HWE in one to three populations ($P < 0.01$) (Table 1).

3.2 Genetic structure STRUCTURE analysis revealed a maximum log-likelihood of posterior probability of the genetic data at $K = 2$ (Figure 1), the proportions of ancestry assigned to different clusters were over 98% for each individual. The Sangzhi population and the rest four populations could be clearly separated (Figure 2).

4. Discussion

The deviation of microsatellites from HWE is possibly due to small sample size or biased sampling, genetic drift, or even poor primer annealing due to nucleotide sequence divergence (e.g., point mutations or indels) (Dakin and Avise, 2004). In this study, three loci (CIBmf01, CIBmf07, and CIBmf14) deviated from HWE in one population, but not in the other four populations, meanwhile their observed heterozygosities were slightly lower than the expected heterozygosities (0.75 *vs*. 0.84, 0.64 *vs.* 0.84, 0.77 *vs*. 0.86), which may result from small sample sizes or biased sampling but further testing is needed. The loci CIBmf05 and CIBmf09 deviated from HWE in no less than two populations and should be carefully used for population genetic study.

Botstein *et al*. (1980) classified loci as highly informative (PIC > 0.5), reasonably informative (0.25 < PIC < 0.5), or slightly informative (PIC < 0.25). According to this standard, 13 of the 15 microsatellite loci in this study are highly informative, and the remaining two loci (CIBmf12 and CIBmf13) are reasonably informative. PIC value on average is 0.678, indicating that *M. fissipes* has high genetic diversity, which may be corresponding to its behavioral adaption in reproduction.

The Sangzhi population of *M. fissipes*, which is outside of the Sichuan Basin, can be clearly separated from the four populations in the Sichuan Basin (Figure 2). This shows that the genetic divergence of this wide-ranging species could be affected by topographic barriers, especially mountain isolation, and this could be tested through thorough sampling across its range.

To sum up, 13 loci obtained in this study were proven to be polymorphic, and useful to clarify the landscape genetics, speciation, and paternity of this species.

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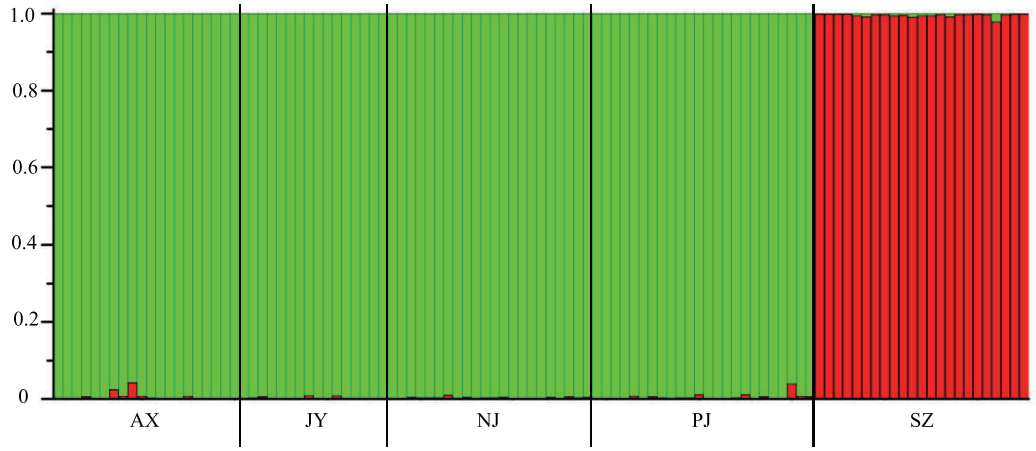

Figure 2 Bayesian STRUCTURE output of two genetic clusters identified (K = 2), represented by two colors: green and red. The proportions of ancestry assigned to different clusters were plotted by individuals from the five sites: Anxian (AX), Jianyang (JY), Nanjiang (NJ), Pujiang (PJ) and Sangzhi (SZ).

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